Many bacterial populations harbour substantial numbers of hypermutable bacteria, in spite of hypermutation being associated with deleterious mutations. One reason for the persistence of hypermutators is the provision of novel mutations, enabling rapid adaptation to continually changing environments, for example coevolving virulent parasites. However, hypermutation also increases the rate at which intraspecific parasites (social cheats) are generated. Interspecific and intraspecific parasitism are therefore likely to impose conflicting selection pressure on mutation rate. Here, we combine theory and experiments to investigate how simultaneous selection from inter- and intraspecific parasitism affects the evolution of bacterial mutation rates in the plant-colonizing bacterium *Pseudomonas fluorescens*. Both our theoretical and experimental results suggest that phage presence increases and selection for public goods cooperation (the production of iron-scavenging siderophores) decreases selection for mutator bacteria. Moreover, phages imposed a much greater growth cost than social cheating, and when both selection pressures were imposed simultaneously, selection for cooperation did not affect mutation rate evolution. Given the ubiquity of infectious phages in the natural environment and clinical infections, our results suggest that phages are likely to be more important than social interactions in determining mutation rate evolution.
mutator frequencies [18,22,23]. Antagonistic coevolution with viruses creates continual selection for novel resistant phenotypes, and has been shown to confer a large selective advantage on hypermutator bacteria in vitro [24,25]. However, hypermutators have been shown to be selected against when cooperative behaviours are advantageous (specifically, the production of siderophores: extracellular iron-scavenging public goods). This is because hypermutators generate non-producing cheats more readily, reducing relatedness in populations founded by mutators [26–28]. Beyond bacteria, selection for cooperation can keep relatedness high through processes such as kin recognition [29,30], reproductive skew [31] and within-group segregation [32].

Here, we combine theory and experiments to investigate how simultaneous selection from inter- and intraspecific parasitism affects the evolution of bacterial mutation rates. We use the well-studied, plant-colonizing bacterium, Pseudomonas fluorescens SBW25, which undergoes extensive reciprocal evolution of defence and counter-defence with an obligatory killing bacteriophage, ϕ2, in both nutrient media [33] and soil [34]. For the cooperative trait, we focus on the extracellular production of iron-scavenging siderophores, which are individually costly to produce but can be used by neighbouring conspecifics. As a result, non-producing cheats can readily invade but reduce iron-dependent population growth rate in iron-limited environments in both P. fluorescens SBW25 [35] and the closely related bacterium, Pseudomonas aeruginosa [36]. We experimentally manipulated these opposing selection pressures by competing P. fluorescens SBW25 and an isogenic mutant in iron-limited or iron-rich media (only the former conditions require siderophore production), and in the presence or absence of phages, in experimental metapopulations. Our experimental design simulated global competition, whereby all patches within a metapopulation were mixed and clones from this mixture were used to inoculate new patches. In doing so, genotypes from the more successful patches were overrepresented in subsequent generations, whereas those from less successful patches were underrepresented. Consequently, any phenotype that reduced group productivity was selected against. To ensure selection for cooperation, we regularly established new patches in the metapopulation with single clones (high relatedness) [27,36]. This design not only allowed the benefits of diversity to be realized by hypermutators, but also kept selection for cooperation high.

2. Theoretical model and analysis

We develop a simple mathematical model to make qualitative predictions about the evolution of bacterial mutation rates \( \mu \) in the presence of social cheats and parasites. We assume that higher mutation rates both increase bacterial resistance to parasites and the local abundance of social cheats. We consider a metapopulation composed of a large number of patches, each of which is colonized by a single bacterial strain with mutation rate \( \mu \), with \( 0 \leq \mu \leq 1 \). All strains are assumed to be functional for the cooperative trait, and cheats arise by loss-of-function mutations. While cheats impose a growth cost on cooperative lineages, we assume that cheats are poor colonizers of new patches, and therefore evolutionarily unviable. We assume that the fitness \( w \) of a bacterial strain with mutation rate \( \mu \) is a function of its survival \( S \) and its growth \( G \), such that \( w(\mu) = SG \).

Bacterial survival decreases as parasite density \( \pi \) increases, while it increases as bacterial hosts evolve resistance to parasites. For simplicity of analysis, we assume that the probability that bacterial hosts acquire resistance alleles is equal to their mutation rate \( \mu \), and that parasite-induced mortality decreases linearly with resistance. As a result, the survival cost imposed by parasites on their bacterial hosts is \( (1 - \mu)\pi \), such that the survival of a strain with mutation rate \( \mu \) is \( S = 1 - (1 - \mu)\pi \). Note that in the absence of parasites (\( \pi = 0 \)), or when there is full resistance (\( \mu = 1 \)), survival is assumed to be 1.

We assume that the costs associated with social cheats depend on three factors: (i) the local abundance of cheats; (ii) the intrinsic cost of cheats to cooperators and (iii) parasite negative density-dependent regulation of hosts’ cooperative growth. We assume that the local abundance of cheats is equal to the mutation rate \( \mu \) of the local bacterial strain. Cheats are assumed to impose an intrinsic cost \( c \) on cooperative growth. In addition, cooperative growth may be subjected to density-dependent regulation by parasites, which reduces the impact of cheats on cooperative growth.

We assume that parasite density-dependent regulation of hosts’ cooperative growth is given by \( \alpha \pi \), where \( 0 \leq \alpha \leq 1 \) is the degree of parasite density-dependent regulation. Putting all these factors together, we define the overall growth cost of cheats to cooperative growth as \((1 - \alpha \pi)c\mu\), and the growth of a focal lineage as \( G = 1 - (1 - \alpha \pi)c\mu \). Note that if: (i) cheats are absent (\( \mu = 0 \)); or (ii) cheats do not impose an intrinsic cost on cooperators (\( c = 0 \)); or (iii) there is full density-dependent regulation (\( \alpha = \pi = 1 \)), growth is one, \( G = 1 \). The fitness of a focal strain with mutation rate \( \mu \) is then given by

\[
\frac{w(\mu)}{c} = \left(1 - (1 - \mu)\pi\right)\left(1 - (1 - \alpha \pi)c\mu\right).
\]

Our objective is to determine the evolutionary stable (ES) mutation rate \( \mu^* \) [37,38]. We find that the marginal fitness benefit of a slight increase in the mutation rate is given by \( B(\mu) = \pi(1 - (1 - \alpha \pi)c\mu) = \pi G \). Thus, the marginal benefit increases with increasing parasite density \( \pi \), as long as there is some growth. The marginal fitness cost of a slight increase in the mutation rate is given by \( C(\mu) = (1 - (1 - \mu)\pi)(1 - \alpha \pi)c\mu = S(1 - \alpha \pi)c \). Thus, as long as there is some survival, the growth cost is low if (i) there is strong parasite density-dependent regulation (high \( \alpha \pi \)); and (ii) the intrinsic cost of cheats is low (low \( c \)). The ES mutation rate is the value \( \mu^* \) for which the marginal cost exactly cancels the marginal benefit of a slight increase in the mutation rate, i.e., \( B(\mu^*) = C(\mu^*) \). This is given by

\[
\mu^* = \frac{1}{2}\left(\frac{1}{\pi} - \frac{1}{\pi} - \pi\right).
\]

We find that the ES mutation rate \( \mu^* \) decreases with increasing intrinsic cost of cheats (\( \partial \mu^*/\partial c < 0 \)). By contrast, the ES mutation rate \( \mu^* \) increases with increasing degree of parasite negative density-dependent regulation of hosts (\( \partial \mu^*/\partial \alpha > 0 \)) and with increasing parasite density (\( \partial \mu^*/\partial \pi > 0 \); figure 1).
3. Experimental material and methods

(a) Strains

The *P. fluorescens* strain SBW25 [39] was used as a wild-type, and the strain SBW25 ΔmutL, that has a mutation in the mismatch repair gene mutL, was used as a hypermutator. SBW25 ΔmutL exhibits a spontaneous mutation rate 100 times higher than that of SBW25 and is resistant to the antibiotic gentamicin [24], allowing it to be distinguished from the wild-type. We used the lytic bacteriophage phi 2 (42) [33].

(b) Experimental design

To ensure that each population was colonized by a single strain (i.e. relatedness = 1) at the first transfer into medium, each bacterial culture was grown in 6 ml King’s Medium B (KB; 10 g glycerol, 20 g proteose peptone no. 3, 1.5 g K₂HPO₄·3H₂O, 1.5 g MgSO₄·7H₂O, per litre), for 24 h at 27°C, after which it was diluted with M9 buffer and grown for 36 h on KB agar at 27°C. A single colony could then be selected and further grown in KB medium at 27°C for 24 h. This single-strain culture could then be used to establish populations.

We manipulated phage and iron availability in a fully factorial experiment, setting up each of our four treatments ((i) iron + phage, (ii) iron only, (iii) phage only and (iv) no iron, no phage) in 96-well plates. We used six replicates for each treatment, with each replicate consisting of a metapopulation made up of six patches (wells). Each well was inoculated with 180 μl KB medium, with three wells of each replicate initially inoculated with approximately 10⁷ colony-forming units (CFUs) of clonal wild-type bacteria, and three wells inoculated with approximately 10⁷ CFUs of clonal mutator bacteria. Iron limitation was created by the addition of 100 μg ml⁻¹ human apotransferrin and 20 mM sodium hydrogen carbonate (NaHCO₃) to standard KB medium immediately before use [35]; NaHCO₃ was also added to non-iron-limited treatments. Approximately 10³ phages were added to phage treatments in 10 μl KB and the same volume of KB media added to no-phage wells. Plates were incubated at 27°C under static conditions.

Every second day, aliquots (2 μl) from all four treatments were transferred to new KB medium (± Fe) using a sterile pin replicator. For every four transfers (8 days), 100 μl from each of the six wells within a replica was mixed, simulating global competition [36] and plated out on KB and KB + gentamicin agar to assess frequencies of mutator and wild-type bacteria (see below). Phage was isolated from this mixture each time, by adding 10% chloroform and centrifuging at 13 000 r.p.m., which lysed and pelleted bacteria. Six colonies from each KB agar plate were randomly selected, grown independently for 24 h in 180 μl KB medium at 27°C and each was subsequently used to inoculate a new well within a treatment (10⁷ CFUs), to ensure high relatedness, and hence the potential for selection for cooperation was continually re-established. For phage treatments, both sympatric (10 μl) phages and ancestral (10 μl) phages were added (to ensure continual selection for phage resistance where sympatric phages had reached low densities); equivalent volumes of phage-free KB media were added to no-phage treatments. Mixing and plating were performed five times in total, for approximately 250 generations of bacterial growth (carrying out six transfers instead of four between mixing after the third assay).

(c) Assays

The relative fitness of mutator and wild-type strains was assessed by plating a mixture of all patches within a replicate onto both KB agar (for an estimate of total density) and KB agar supplemented with 10 μg ml⁻¹ gentamicin (on which only gentamicin-resistant mutator bacteria can grow) over 36 h, resulting in a KB and KB + gentamicin plate for each replicate and six plates of each type per treatment. Colony types were also categorized by colour into either siderophore producers (yellow/green) or non-producers (white), based on the fact that the primary siderophore of *P. fluorescens*, pyoverdine, is yellow/green. As the amount of colony pigmentation varied considerably, we classified a colony showing any yellow/green pigmentation after 36 h as a producer [26]. We carried out an additional experiment to verify that the appearance of milky white colonies indeed represented the evolution of siderophore negative cheats. We selected 19 wild-type and 19 milky colonies at random at the final timepoint, and inoculated each colony into iron-limited KB media. Populations were grown at 27°C for 24 h, after which, fluorescence of 180 μl of culture was measured at 460 nm, following excitation at
400 nm using a Biotek Synergy 2 spectrophotometer (standard protocol for measuring pyoverdine production [40,41]). Milky colonies produced pyoverdine at significantly lower levels than wild-type (Student’s t-test, \(T_{18.012} = 15.7631, \ p < 0.0001\)), and so any milky colonies that emerged during the experiment were labelled siderophile cheats.

(d) Costs of cheating and phage parasitism
To directly assess the relative cost of cheats and parasites on the growth of wild-type bacteria, wild-type SBW25 and an isogenic cheat strain (SBW25\(\Delta\)pvdL, with the primary sidero-

phore, pyoverdine, knocked out, [42]) were grown in isolation in the presence and absence of phages (\(\phi\)2 (\(\phi\)2)), with eight replicates per treatment. All treatments were carried out in iron-limited KB broth to facilitate selection for cooperation under starting conditions described above. After 24 h at 27°C, the abundance of cheat and wild-type genotypes was assessed by plating each treatment on KB agar, incubating for 36 h, and counting the colonies on each plate as before. We also confirmed a previous work [35] that cheats have a growth rate advantage in the presence of wild-type bacteria under these conditions by competing 50:50 mixtures of cheats and wild-type. Experimental data and R codes are available from the dryad repository: doi.org/10.5061/dryad.tq34k.

(e) Statistical analysis
R software [43] was employed for all statistical analyses. Maximum-likelihood analysis was carried out using Linear Mixed-Effects Revised models with arcsine square root (proportion of mutators/cheats) as a response variable, and iron, phage and time, including two-way interactions, as explanatory variables. A third model with cheat frequency as the response variable was employed to investigate any affect mutator frequency might have on the appearance of cheats and the extent to which this was affected by iron limitation. Iron and phage presence/absence were fitted as factors and time as a continuous variable, assigning ‘population’ as a random factor as follows:

\[
\text{mutator frequency} \sim \text{iron} \times \text{phage} + \text{iron} \times \text{time} \\
+ \text{phage} \times \text{time} + 1|\text{population} \\
\text{cheat frequency} \sim \text{iron} \times \text{phage} + \text{iron} \times \text{time} \\
+ \text{phage} \times \text{time} + 1|\text{population} \\
\text{cheat frequency} \sim \text{mutator frequency} \times \text{iron} + 1|\text{population},
\]

where \(\times\) represents an interaction and separate terms. To assess the relative costs of cheating and parasitism, Student’s t-tests were used to compare fitness of wild-type and cheat strains after 24 h growth in the presence and absence of phage.

4. Experimental results
(a) Costs of cheats and phages
To establish the relative magnitude of costs of cheating and phage parasitism on bacterial growth, we measured the densities of siderophore cheat and wild-type strains grown as monocultures in the presence and absence of ancestral bacteriophages after 24 h growth under iron-limited conditions. Phages caused a significant reduction in the density of wild-type (Student’s t-test, \(T_{7.515} = 5.6787, \ p < 0.001\)) and cheat

phenotypes (Student’s t-test, \(T_{11.542} = 2.4999, \ p < 0.05\)). Consistent with previous work [35,42], cheats reached significantly lower densities than wild-type bacteria in the absence of phages (Student’s t-test, \(T_{8.016} = 4.6885, \ p < 0.001\)), however, in the presence of phages, this relationship was reversed and cheats grew significantly more than wild-type (Student’s t-test, \(T_{11.124} = 3.3046, \ p < 0.01\)). The reduction in the density of wild-type bacteria brought about by cheats was significantly smaller than the reduction caused by the presence of phages (Student’s t-test, \(T_{8.677} = 4.5904, \ p < 0.01\)).

Figure 2. Changes in mutator frequency in a population through time is affected by the presence/absence of phage (\(\chi^2_{1,5} = 9.3343, \ p < 0.01\)). Data are plotted using predictions from minimal LMER model (mutator frequency \(\sim\) time: phage + 1|population).

(b) Competition between mutators and wild-type over evolutionary time scales
We predicted that phages should select for higher mutation rates, while selection for cooperation should select against mutators. Phage-imposed selection was manipulated by the presence versus absence of phages, whereas selection for siderophore-mediated cooperation was manipulated by changing iron availability (siderophores are required when iron is limited) in experimental metapopulations. While the frequency of mutators increased through time in the presence of phages and decreased in the absence of phages (LMER: timepoint \times phage interaction, \(\chi^2_{1,5} = 9.3343, \ p < 0.01\); figure 2) iron availability had no impact on the frequency of mutators (LMER: \(\chi^2_{1,5} = 0.3097, \ p = 0.58\)). Moreover, there was no interaction between the presence of phage and iron availability (LMER: non-significant phage \times iron interaction, \(\chi^2_{1,5} = 0.187, \ p = 0.67\)). Selection for mutators via the presence of external viruses, therefore, was not affected by any conflicting selection for cooperation by iron limitation.
predictions from minimal LMER model (cheat frequency ≈ \( \frac{1}{C^2} \) non-significant phage presence of phages in determining cheat frequency (LMER: no interaction was observed between iron availability and the consistent with previous work using this system [22,24,25].

Here, we investigate both theoretically and empirically how selection imposed by interspecific parasites (bacteriophages) and intraspecific parasites (social cheats) affects the evolution of mutation rates in bacteria. Theoretically, selection for mutator bacteria decreases when there is selection for cooperation because mutators are more likely to generate phage resistance mutations. Our short-term growth rate experiments suggest that phages impose a much greater growth rate cost than social cheating in this experimental context. Moreover, the impact of phages on density was less for cheat than wild-type populations. Feeding this back into our simple model suggests that the strength of phage-imposed positive selection on mutators will be greater than cooperation-imposed negative selection on mutators. Our evolution experiment was consistent with this prediction: mutator frequency increased in the presence of phages but was not influenced by whether or not there was selection for cooperation.

That coevolution with phages favours mutator bacteria is consistent with previous work using this system [22,24,25].

Pal et al. [24] found that after approximately 170 bacterial generations, mutation rates had increased 10- to 100-fold in nine out of 36 populations in the presence of coevolving phages, whereas no significant change in mutation rate was observed in populations evolving in the absence of phages. Qualitatively identical results were observed when the wild-type and an isogenic mutator were competed at equal ratios in the presence and absence of phages. However, in these experiments bacteria were cultured as single populations. Our results reveal similar phage-imposed selection for mutation rates in high-relatedness metapopulations. Arguably, such metapopulation structure presents a more ecologically relevant scenario for bacteria, where microcolonies are founded by single bacteria, which then go extinct or go on to colonize new patches [44].

The lack of association between selection for siderophore-cooperation and mutator frequency is initially surprising, as a previous study showed such a relationship using the related bacterium, P. aeruginosa [27]. Specifically, in an evolution experiment of a similar duration to this study (approx. 250 generations), mutant frequency was significantly lower in iron-limited metapopulations when patches were established with single colonies (high relatedness, hence strong selection for cooperation) compared with multiple colonies. In both studies, cheats of both species show reduced growth under iron-limited conditions (this study, [36,42,45]). The discrepancy between these results is likely to have arisen because (i) different species and media were used, and hence the cost of cheating may be different, (ii) selection for cooperation in this study was manipulated by iron availability as opposed to number of colonies founding a patch and (iii) we used the mutL mutator strain rather than mutS as in previous studies [24,27], and differences in genetic effects of the specific gene may account for discrepancies. However, studies in Escherichia coli suggest these mutations have very similar effects [46].

Our model and ecological experiment suggests a simple but potentially general interaction between social cheating and parasitism: parasites have less of an impact on reducing cheat population density, and hence the relative impact of cheating on population growth is reduced in the presence compared with the absence of parasites. This is a simple extension of the more general finding that enemies typically reduce high-density populations relatively more than low-density populations [47,48]. In addition to weakening selection against mutators, this reduced costs of cheats also suggests that cheats may be relatively favoured in the presence versus absence of parasites. By contrast, recent theory and experiments suggest cheating may be less favoured in the presence of parasites, because cheats are likely to be initially rare in newly colonized patches, and hence less likely to evolve resistance mutations [35,49]. Both mechanisms are not mutually exclusive, with the former relevant when cheats are at sufficiently high frequency to affect growth and the latter when cheats are initially rare. The simultaneous operation of both mechanisms may explain our finding that phages had no net effect on the presence of cheats.

High mutation rates can result in rapid resistance to antibiotics and bacteriophage, and clinical studies have shown hypermutators to be associated with poorer lung function in CF patients [50]. Therefore, it is crucial to develop an understanding of how different selection pressures involved in their persistence interact with each other. Given
the ubiquity of infectious phages in the natural environment [51] and clinical infections [52,53], our results suggest that phages are likely to be more important than social interactions in determining mutation rate evolution in natural environments.

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